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Safety evaluation of glucose oxidase from *Penicillium chrysogenum*

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ABSTRACT

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase; EC 1.1.2.3.4) is used in the food and beverage industry as a preservative and stabilizer and is commonly derived from the fungus *Aspergillus niger*. Although the safety of glucose oxidase preparations from *A. niger* is well-established, the use of preparations derived from other fungal species is of interest; however, an assessment of their safety is warranted. Here, we report on the safety of a glucose oxidase preparation derived from the fungus *Penicillium chrysogenum* (designated as PGO) for commercial use in food processing, as well as an ingredient in food. In a repeated dose 90-day oral toxicity study conducted in rats, PGO was without compound-related adverse effects at doses of up to 15,600 U/kg body weight/day, equivalent to 193 mg total organic solids/kg body weight/day. In addition, PGO was non-genotoxic in a series of genotoxicity tests, including a bacterial reverse mutation test, an *in vitro* mammalian chromosomal aberration test, and a combined *in vivo* mammalian erythrocyte micronucleus test and comet assay. The results of these studies support the safe use of PGO in food for human consumption.

1. Introduction

Commercial application of glucose oxidase in a variety of industries has been on the rise in recent years. Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase; EC 1.1.2.3.4) is a well-characterized enzyme that catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone in the presence of molecular oxygen, which serves as an electron acceptor. D-Glucono-δ-lactone is subsequently hydrolyzed to gluconic acid by non-enzymatic means. Hydrogen peroxide (H_2O_2) also is produced as a by-product of the reaction. Of interest is the application of glucose oxidase in the food and beverage industry. Applications of glucose oxidase in these industries include use as a preservative and stabilizer. The enzyme, in conjunction with catalase, is often applied to remove residual glucose and oxygen in foods and beverages to prolong shelf-life (as reviewed by Wong et al., 2008 and Bankar et al., 2009). For instance, glucose oxidase is used to remove glucose during the manufacture of egg powders and during baking and fruit processing to prevent non-enzymatic browning induced by the Maillard reaction (Crueger and Crueger, 1990; Sisak et al., 2006). The enzyme also has been used to remove oxygen to prevent color and flavor loss in canned foods and beverages, such as beer and soft drinks (Crueger and Crueger, 1990; Bankar et al., 2009). In addition, glucose oxidase is used to strengthen gluten contained in wheat flour-based starch products, such as breads and other baked products, thereby improving quality (Vemulapalli and Hoseney, 1998; Vemulapalli et al., 1998; Rasiah et al., 2005). The latter is achieved *via* increased formation of crosslinks between wheat proteins, including between albumin and globulin and between gluten (glutenen) proteins (Rasiah et al., 2005). Glucose oxidase may also improve bread quality by decreasing the sulfhydryl content and increasing viscosity in the water soluble portion of dough (Vemulapalli and Hoseney, 1998).

Glucose oxidase used in commercial applications of food processing is obtained from naturally producing fungal sources, most commonly from the mycelium of *Aspergillus niger* (Bankar et al., 2009). *Penicillium* species also produce glucose oxidase, and of particular interest is the commercial use of glucose oxidase purified from the filamentous fungus *Penicillium chrysogenum*. Although glucose oxidase derived from *A. niger* has a history of safe use in

Abbreviations: 2-AA, 2-aminoanthracene; 9-AA, 9-aminoacridine hydrochloride; A/G, albumin:globulin; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BUN, blood urea nitrogen; bw, body weight; DNA, deoxyribonucleic acid; EDTA-2K, ethylenediaminetetraacetic acid-2K; γ-GGT, γ-glutamyl transpeptidase; GLP, good laboratory practice; H_2O_2, hydrogen peroxide; HCl, hydrogen chloride; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MI, mitotic index; NaN_3, sodium azide; NaOH, sodium hydroxide; OECD, Organisation of Economic Co-operation and Development; PGO, *Penicillium chrysogenum*; PT, prothrombin time; RBC, red blood cell; U, units; WBC, white blood cell.

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the food industry, the safe use of glucose oxidase derived from the production strain *P. chrysogenum* has not yet been formally established. *P. chrysogenum* has a history of safe use in the production of food for human consumption (Mogensen et al., 2002), and therefore, would be a suitable candidate for use as a source strain in the production of microbial-derived enzyme preparations. The objective of this study was thus to specifically evaluate the safety of a glucose oxidase preparation derived from *P. chrysogenum* strain PGO 19–162 (referred herein as PGO) for use in food processing. To this end, PGO was subjected to standard toxicological tests, consisting of a 90-day repeated oral toxicity test conducted in rats and a series of genotoxicity tests, including a bacterial reverse mutation test, an *in vitro* mammalian chromosomal aberration test, and a combined *in vivo* mammalian erythrocyte micronucleus test and comet assay.

2. Materials and methods

2.1. Enzyme preparation

The Sumizyme PGO enzyme preparation (Lot No. 090506R), produced as described below, was provided by Shin Nihon Chemical (Anjyo, Aichi, Japan) as a brown, odorless, transparent stock solution. The same lot was used in all studies[1]. Analysis of the test article confirmed that the test article used in the studies met the product specifications. The concentration of PGO in the stock solution was 1560 units (U)/mL, with the amount of test article per unit of enzyme activity providing 0.0124 mg of total organic solids (TOS) (i.e., 0.0124 mg TOS/U). The test article was stored in an airtight container in a freezer at −24.1 to −15.2 °C, and was analytically determined to be enzymatically stable throughout the experimental period for all studies.

Briefly, the PGO enzyme preparation was prepared by standard culture methods using *P. chrysogenum* strain PGO 19–162 as the production strain. *P. chrysogenum* strain PGO 19–162 was originally isolated from soil based on its glucose oxidase properties. The strain has been deposited in the CABI Bioscience collection (IMI No. 390502). Following cultivation, the *P. chrysogenum* culture was subjected to multiple filtration and ultrafiltration steps to remove the production strain and other materials (*e.g.*, proteins, saccharides, lipids, salts, and other compounds less than 5000 MW). Ethanol was added to the filtered solution to precipitate the enzyme, and the resultant slurry was washed with ethanol three times. The slurry was then centrifuged to isolate the precipitated solid enzyme material. The solid precipitate was dried by vacuum and the dried enzyme preparation was powdered by passing through a mesh sifter. The final PGO enzyme preparation was analytically determined to be devoid of any chemical (*e.g.*, heavy metals) or microbiological contamination (*e.g.*, bacteria, including coliforms, *Escherichia coli*, and *Salmonella* species, and mold).

2.2. Repeated dose 90-day oral toxicity study

This study was conducted in accordance with the OECD Guideline for the Testing of Chemicals No. 408 (OECD, 1998b) and the Guideline for Designation of Food Additives and for Revision of Standards for Use of Food Additives (Ministry of Health, Labor and Welfare of Japan, 1996). Further methodological details are provided below.

[1] All studies were conducted at Biosafety Research Center (formally, Biosafety Research Center, Foods, Drugs, and Pesticides), 582-2, Shioshinden, Iwata, Shizuoka 437-1213, Japan. Biosafety Research Center is a GLP-compliant testing facility. All studies were performed in compliance with the Organisation of Economic Co-operation and Development (OECD) principles of good laboratory practice (GLP) (OECD, 1998a),

2.2.1. Preparation of dosing formulations

PGO dosing formulations were prepared on each administration day by serially diluting the thawed stock solution (1560 U/mL) by 10-fold with distilled water (Japanese Pharmacopoeia grade) (Otsuka Pharmaceutical Factory, Japan) to obtain the mid- and low-dose formulation concentrations of 156 and 15.6 U/mL, respectively. The undiluted stock solution served as the high-dose formulation. Dose formulations were stored on crushed ice until administration.

2.2.2. Animals and treatment

Forty-five (45) male and 45 female 4-week-old Crl:CD(SD) (SPF) rats were obtained from Charles River Laboratories Japan (Atsugi, Kanagawa, Japan). Upon receipt, animals were quarantined and allowed to acclimate to the laboratory environment for eight consecutive days. Animals were housed individually in aluminum cages with stainless steel wire mesh fronts and floors hung in an automatic water flushing breeding rack. Housing conditions were maintained at a temperature of 23 ± 3 °C and a relative humidity of 55 ± 20%, with 12 or more fresh air ventilations/h and a 12-h light/dark cycle. The cages were exchanged every 2 weeks and feeders exchanged once a week. Animals were provided an irradiation-sterilized commercial diet (CRF-1; Lot No. 100203, Oriental Yeast, Tokyo, Japan) and tap water (Iwata municipal tap water) *ad libitum*. All contaminants in the feed and water were analytically confirmed to be within acceptable ranges.

During the acclimatization period, animals were observed daily for signs of general health and body weights were measured on the day of receipt (day-8) and on the last day of the acclimatization period (day-1). Ophthalmological examination also was performed on all animals at the end of the acclimatization period. Forty (40) rats of each sex that were in good general health and with good ophthalmology results were selected and randomly assigned to one of four groups (10 rats/sex/group) based on body weights measured on the first day of dose administration (*i.e.*, day 1 of the administration period) using a computer system package developed for safety studies (LATOX-F, FFC, Tokyo, Japan). On the first day of dosing, animals were 5 weeks of age with males weighing 159–184 g and females weighing 124–147 g.

PGO doses to be administered in the 90-day study were selected based on the results of a previous 2-week dose-range finding study performed in the same strain of rats and under equivalent study conditions. In the dose-range finding study, groups of five rats per sex were administered PGO at doses of 156, 1560, or 15,600 U/kg body weight (bw)/day by oral gavage, equivalent to 1.93, 19.3, and 193 mg TOS/kg bw/day. Control animals were administered vehicle control (distilled water). No compound-related adverse effects were observed following administration of PGO, and thus, 15,600 U/kg bw/day PGO was selected as the high dose to be administered in the 90-day study.

In the 90-day study, groups of 10 male and 10 female rats were administered PGO at a dose of 156 (low-dose), 1560 (mid-dose), or 15,600 (high-dose) U/kg bw/day by oral gavage using a Teflon gastric tube and disposable syringe once daily for a period of 90 days. The control group consisted of 10 males and 10 females administered the vehicle control (distilled water) by the same method. The dosing volume administered to each animal was set at 10 mL/kg body weight and the actual administration volume was calculated for each individual animal on the basis of the most recent body weight.

2.2.3. Clinical observations, body weights, food consumption, and opthalmology

All animals were observed for clinical signs and mortality at least twice daily; before and after dosing (except clinical signs were recorded once on the day of necropsy). Body weights were